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Serial No.: 09/804,409

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Applicant	:	Kieffer et al.	Art Unit	:	1632
Serial No.	:	09/804,409	Examiner	:	Kelly, Robert M.
Filed	:	03/12/2001			
Title	:	COMPOSITIONS AND METHODS FOR REGULATED PROTEIN EXPRESSION IN GUT			

Assistant Commissioner of Patents
Washington, DC 20231

DECLARATION OF DR. ANTHONY CHEUNG UNDER 37 C.F.R. §1.132

Sir:

1. I, ANTHONY CHEUNG, Ph.D., declare and say I am a resident of Vancouver, British Columbia, Canada. My residence address is: 1127 E. 16th Ave., Vancouver, BC, Canada V6E 4M4. I received Bachelor of Science degree in Biochemistry from the University of British Columbia in 1993. I received a Doctor of Philosophy degree in Physiology from the Tulane University in 1999. I am currently Chief Scientific Officer of enGene, Inc., in Vancouver, BC, Canada. My curriculum vitae is attached, which reflects my expertise in the areas of molecular biology and biochemistry.
2. I am an inventor of the subject matter claimed in United States Patent Application Serial No. 09/804,409, filed March 12, 2001.
3. I have reviewed the claims that are presently under examination.
4. I understand that claims 31 to 55 remain rejected due to an alleged lack of enablement for *in vivo* gene therapy.
5. I submit this declaration and the studies herein to further corroborate that a polynucleotide encoding a protein can be introduced into mucosal tissue cells *in vivo* and the expressed protein is produced in animals in an amount effective to treat a disorder treatable by producing the protein, without undue experimentation. I also submit this declaration and the studies herein as evidence that different gut endocrine promoters can be used to drive production of protein at therapeutically relevant levels following *in vivo* delivery of a polynucleotide encoding the protein to mucosal tissue of animals.

Exhibit AA

6. Recombinant feline immunodeficiency virus (FIV) vector particles were packaged by co-transfecting 293T cells with an established triple-plasmid calcium phosphate transfection protocol. Polynucleotides encoding the proteins were an FIV vector harboring either a human insulin gene or the secreted embryonic alkaline phosphatase (SEAP) gene, which were driven by a rat GIP promoter or a mouse chromagranin A promoter, respectively. The other two plasmids were 1) an FIV packaging construct expressing FIV gag and pol genes; and 2) an envelope construct expressing VSV-G envelope glycoprotein.
Briefly, 293T cells ($1.5x10^7$) were transfected with 8 μg of packaging plasmid, 4 μg of envelope plasmid and 8 μg of vector plasmid in each 150 mm cell culture dish. Eight hours after transfection, cells were fed with fresh media (Dulbecco Modified Eagle media with 10% FBS) and incubated at 37°C overnight. The culture media was replaced the next morning, transfected cells were transferred to a 32°C incubator and virus particles were harvested from the culture media at 48 h and 72 h post-transfection, concentrated by centrifugation at 50,000g for 2 hours and stored in TNE buffer (50 mM of Tris and 130 mM of NaCl and 1mM of EDTA) at -80°C until use.
FIV vector titers were determined by real-time quantitative PCR (RQ-PCR) using primers and fluorescent probes specific for the gene-of-interest. A9L mouse fibroblast cells ($5x10^5$) were infected with 5 μl of FIV virus and the genomic DNA of infected cells isolated 48 hours later with a Qiagen DNAeasy Tissue Kit (Qiagen, Chatsworth, CA).

7. *In vivo* delivery of viral vectors was performed with male C57/B16 mice (Jackson Laboratories), which were housed in a 12h light/dark cycle and fasted overnight prior to the procedure for viral vector delivery. An abdominal incision was made in anesthetized animals and a section of duodenum (~2 cm) was lifted from the abdominal cavity using a glass hook. A tourniquet was place around the pyloric sphincter and the isolated section of the duodenum was washed once with warm saline followed by a 10 min incubation with ~0.12 ml of 0.2% n-dodecyl-beta-D- maltoside (DDM). The duodenum was then washed twice with ~150 μl of warm saline and the isolated section of the duodenum was maintained in an elevated position. The viral vector was delivered to the lumen of the duodenum by a single injection, which was allowed to incubate in the elevated duodenum

section for 1 hour. After incubation, the tourniquet was released, the duodenum returned to the abdominal cavity and the incision closed.

8. To determine C-peptide and SEAP levels in animals, blood samples were collected in heparinized capillary tubes by saphenous vein bleeding and transferred to a microcentrifuge tube containing 3μL Trasylol. Plasma was isolated from the blood samples by centrifugation and stored at –80°C until assayed.
Plasma levels of human C-peptide, a by-product of insulin biosynthesis, were performed using a commercially available ELISA kit, according to the manufacturer's instructions (Alpco). The antibodies used in the ELISA specifically recognize the C-peptide of human origin and have very low cross reactivity with mouse C-peptide (<0.08%).
Plasma levels of recombinant human SEAP were measured using a commercially available chemiluminescence SEAP Reporter Gene Assay kit (Roche), according to the manufacturer's instruction. Standard curves were generated using human placental alkaline phosphatase (Sigma). Luminescence signals were quantified with a Molecular Devices Lmax II^{384} luminometer.

9. Destruction of insulin-producing pancreatic β-cell was induced by streptozotocin (STZ), a pancreatic β-cell-specific toxin. For these studies, STZ was administered to animals via an intraperitoneal injection (200 mg/kg body weight in citrate buffer). At this dose of STZ, mice typically display glucosuria within 2 days post injection. If left untreated (without administering exogenous insulin), animals typically lose body weight rapidly and die from acute diabetes within days after STZ treatment.

10. To demonstrate that administration of viral vectors to mucosal tissue cells in duodenum leads to production of proteins at therapeutic levels in the bloodstream, FIV vectors carrying human insulin gene driven by rat GIP promoter were delivered into duodenum *in vivo* as described above (10^8 transduction unit/ animal, n=4). Blood levels of human C-peptide were monitored weekly for up to 150 days after treatment. C-peptide is released from insulin producing cells in equimolar amounts to insulin. Approximately 50 days after vector delivery, plasma human C-peptide levels began to increase and subsequently remained at an average of about 15 pM (Figure 1, attached).

Approximately 120 days after vector delivery levels of plasma human C-peptide began to decrease, which is presumably due to immune clearance of human insulin by the mouse. At 150 days after vector delivery, three of the five animals continued expressing circulating human C-peptide at an average plasma concentration of 9.6 pM, and two showed undetectable levels of human C-peptide. These data indicate that long term production of insulin at therapeutically relevant levels in the bloodstream of animals can be achieved by a single *in vivo* administration of vector harboring a nucleic acid encoding insulin into gut or gastrointestinal mucosal tissue cells.

11. To determine if the insulin produced in the animals is functional as well as therapeutic, mice that continued to produce insulin 150 days after vector delivery and mice that no longer produced C-peptide were injected with a high dose of STZ (200 mg/kg). The three mice that continued producing human C-peptide survived STZ-induced diabetes (red broken line, Figure 2). In contrast, STZ treatment of the two mice that expressed no detectable C-peptide resulted in acute diabetes and the animals died within 3 days after STZ treatment (black solid line, Figure 2). These data indicate that the insulin delivered by *in vivo* administration of a nucleic acid encoding insulin into gut or gastrointestinal mucosal tissue cells is A) functional and; B) is produced at therapeutic levels.

12. To further demonstrate that proteins in general can be produced at therapeutically relevant levels by gut or gastrointestinal mucosal cells transformed with a polynucleotides encoding the proteins, a different promoter, namely mouse chromagranin A promoter, was used to drive expression of SEAP. In mice delivered FIV vectors carrying a mouse chromagranin A promoter driven SEAP gene, SEAP protein was detected in the circulation by 5 days after delivery. Plasma levels of SEAP began to plateau at day 10 and remained relatively constant at an average plasma concentration of approximately 15 pg/ml until the end of the monitoring period, at day 58 (Figure 2). Circulating SEAP concentrations were in the pM range, as was the human C-peptide. These data indicate that proteins in general can be produced by delivering a nucleic acid encoding the proteins into gut mucosal tissue cells *in vivo* can be produced at therapeutic levels.

13. Levels of insulin and SEAP achieved by the studies described herein would be considered therapeutic for other proteins. For example, plasma insulin as low as 10-15 pM is sufficient to maintain normal glycemia in diabetic rats (see, for example, Olson et. al. Human Gene Therapy 14:1401 (2003)). In addition, the therapeutic level of glucagon-like peptide-1 (GLP-1) is within 10-20 pM range (Holst and Deacon, Diabetes 47:1663 (1998)). Thus, levels of circulating proteins produced as described herein are therapeutically relevant for these and can reasonably be expected for proteins in general.

14. Based upon the foregoing *in vivo* data, different proteins can be produced at therapeutic levels *in vivo* when vectors harboring nucleic acid encoding the proteins are delivered to gut or gastrointestinal mucosal cells of animals. Furthermore, these studies indicate that therapeutic levels of protein can be produced in animals over a long time period and with a single administration *in vivo*. Moreover, the studies described herein indicate that systemic delivery of proteins at therapeutic levels can be achieved with different promoters that target expression of the protein to gut or gastrointestinal mucosal cells.

15. In view of the foregoing *in vivo* studies and the previous *in vivo* studies in the record, I conclude that transfer of a nucleic acid encoding a protein into gut or gastrointestinal mucosal tissue cells *in vivo* and production of the protein at therapeutically relevant levels in animals would not have required undue experimentation.

16. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Feb. 08, 2005
Date

ANTHONY CHEUNG, Ph.D.

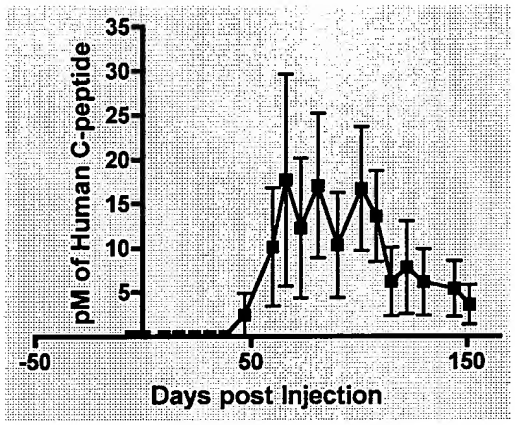

Figure 1: Plasma human C-peptide levels in FIV-rGIP/hIns-treated mice. One hundred and twenty five µl of vector solution (~10^8 transduction units per animal) was incubated in the lumen of the duodenum of CD-1 mice (n=5) as described in the Declaration. Data shown are mean ± SEM.

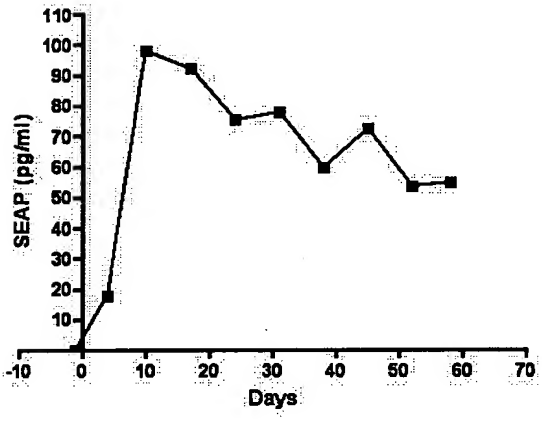

Figure 2: Levels of circulating human C-peptide achieved in FIV-rGIP/hIns-treated mice protected the animals from the lethality of acute diabetes induced by STZ treatment. Mice with detectable circulating human C-peptide (n=3, mean plasma C-peptide = 9.6 pM) survived the high dose STZ treatment. However, mice with no detectable human C-peptide died within 3 days after STZ treatment (black line, n=2).

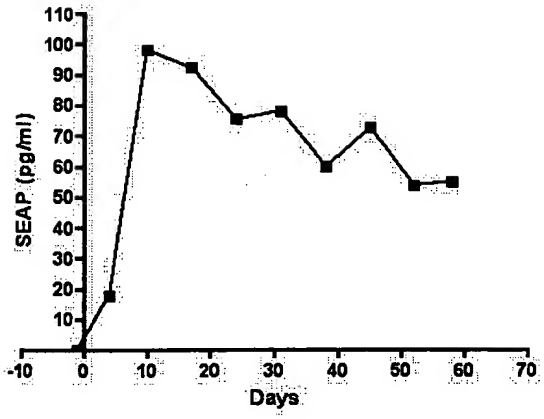

Figure 3: Plasma SEAP concentrations in FIV-mCgrA/SEAP-treated mice. On day 0, 125 ml of vector solution (~10^8 transduction units per animal) was delivered to the lumen of the duodenum of CD-1 mice (n=2) as described in the Declaration.